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Applicant	:	Weinberger et al.
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For	:	EFFECT OF BDNF GENOTYPE ON HIPPOCAMPAL FUNCTION AND VERBAL MEMORY AND RISK FOR SCHIZOPHRENIA
Examiner	:	Sitton, Jehanne Souaya
Group Art Unit	:	1634

IN RE KATZ DECLARATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Daniel R. Weinberger, M.D. do declare and say that:

1. I am a co-inventor of the above-identified application.

2. This declaration is to establish that Hariri et al., Program No. 620.12 Abstract Viewer/Itinerary Planner, Washington, DC, Society for Neuroscience, 2002, 8/19/2002, is a publication of applicant's own invention.

3. The named inventors are Michael F. Egan, Bhaskar S. Kolachana, David Goldman, Joseph H. Callicott, Terry E. Goldberg, and me. We examined the effects of a valine (val) to methionine (met) substitution in the 5′ pro-region of the human brain-derived neurotrophic factor (BDNF) protein. In human subjects, the met allele was associated with impaired hippocampal dependent verbal memory, assayed with memory scores, hippocampal

activation, assayed with functional magnetic resonance imaging (fMRI), and hippocampal n-acetyl aspartate (NAA), assayed with magnetic resonance imaging (MRI) spectroscopy. While we found no relationship between BDNF genotype and schizophrenia, our results demonstrate that BDNF and its val/met polymorphism plays a role in hippocampal function and hippocampal dependent verbal memory in humans. We published these results as Egan et al., Cell 112:257 (Jan 2003).

4. We contributed our expertise essentially as follows: Joseph H. Callicott in measuring hippocampal function, Terry E. Goldberg in measuring verbal memory, Michael F. Egan in measuring verbal memory and providing analysis, Bhaskar S. Kolachana in developing assays, David Goldman in medical informatics, and myself in the ideation to search gene databases for variation in BDNF that might be linked to schizophrenia or other derangements in hippocampal function.

5. The authors of Hariri et al., Program No. 620.12 Abstract Viewer/Itinerary Planner, Washington, DC, Society for Neuroscience, 2002, 8/19/2002, who are not named as co-inventors are A.R. Hariri and V.S. Mattay. Both were collaborators who were involved only with assay and testing sufficient to be listed as co-authors but are not considered co-inventors.

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I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Respectfully submitted,

Dated: 12/14/06

By: _____
Daniel R. Weinberger, M.D.

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ORIGINAL ARTICLE

Cognitive Impairments in Patients With Schizophrenia Displaying Preserved and Compromised Intellect

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Background: Although intellectual and neurocognitive deficits accompany schizophrenia, there are inconsistencies in the literature concerning issues of intellectual decline, premorbid deficits, a modal deficit pattern, and preserved abilities.

Methods: A battery of neuropsychological tests was administered once to 117 consecutively admitted patients with chronic schizophrenia and a group of 27 healthy control subjects to examine patterns of premorbid and current intellect (measured by means of reading scores and IQ, respectively) and the attendant cognitive profiles in schizophrenia using classification methods based on clinically derived (IQ levels) and atheoretical (cluster) techniques.

Results: Sixty patients (51%) with schizophrenia who displayed a general intellectual decline of 10 points or greater from estimated premorbid levels also exhibited deficits of executive function, memory, and attention. Twenty-eight patients (23%) with consistently low estimated premorbid intellect and current intellectual levels who displayed no evidence of IQ decline exhibited language and visual processing deficits in addition to deficits present in the intellectually declining group. The remaining 29 patients (25%) who displayed average estimated premorbid intellectual levels did not show IQ decline and exhibited a cognitive profile similar to normal, with the exception of executive function and attention impairment. Atheoretical analyses support the findings from clinically derived subgroups.

Conclusions: These results suggest that IQ decline, although modal in schizophrenia, is not universally characteristic and that executive function and attention deficits may be core features of schizophrenia, independent of IQ variations.

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SCHIZOPHRENIA HAS been characterized by executive function, attention, memory, and general intellectual deficits. Intellectual decline may occur subsequent to onset of schizophrenia.[1-3] In monozygotic twin pairs discordant for schizophrenia, Goldberg et al[4] demonstrated a 10-point IQ discrepancy in favor of the unaffected twin. Longitudinal studies support intellectual decline,[5,6] as do studies of first-episode patients.[1]

However, intellectual declines after the onset of schizophrenia are not universal.[7] Russell et al[8] suggested that any subsequent intellectual deficit is due to an early decline that predates onset of schizophrenia. In fact, the schizophrenia literature is replete with evidence of low premorbid function.[9] Numerous reports document low premorbid IQ in children who later develop schizophrenia.[3,10-15] Large birth cohorts have shown subtle but significantly lower premorbid levels of educational achievement, retardation in attainment of neuromotor developmental milestones, premorbid speech abnormalities, or intellectual diminution in individuals who later develop schizophrenia.[16,17] Other large population-based cohorts[18,19] reported that patients with schizophrenia displayed higher frequencies of low premorbid ability or IQ. Conversely, some studies have characterized high-functioning patients with schizophrenia who do not display intellectual decline.[20-22] For example, Palmer et al[23] reported that 27% of patients displayed a normal performance on a variety of cognitive measures, including IQ.

In the present study, we assessed a large sample of consecutively admitted patients with chronic schizophrenia. The cognitive domains of memory, attention, executive function, and visual perception were assessed. Premorbid intellect was inferred indirectly using the Wide Range Achievement Test–Revised (WRAT-R) Reading test. We addressed the following questions: (1) Is intellectual decline universally characteristic? (2) What other patterns of intellectual compromise or preservation might be present? and (3) What are the implications for other cognitive domains in patients whose premorbid and morbid intellect varies? Based on previous studies, we hypothesized that a group

SUBJECTS AND METHODS

SUBJECTS

One hundred seventeen patients, 84 males and 33 females, with a diagnosis of schizophrenia who were consecutively admitted to the National Institute of Mental Health Neuroscience Center at St Elizabeths, Washington, DC, participated in this study. The number of patients contributing to the analysis of any particular test outlined herein varied slightly because of patient compliance. A board-certified psychiatrist made the diagnosis by the Structured Clinical Interview for *DSM-III-R* using *DSM-III-R* criteria without knowledge of the neuropsychological evaluations. Patients who received concurrent Axis I psychiatric diagnoses or those who had a history of current substance abuse, head injuries with concomitant loss of consciousness, seizures, central nervous system infection, diabetes, or hypertension were excluded. Patients were classified into undifferentiated (63.3%), paranoid (25.6%), disorganized (9.4%), and residual (1.7%) subtypes. In addition to patients with schizophrenia, 27 healthy control subjects, recruited through the National Institutes of Health Normal Volunteer Office, participated in this study. Healthy control subjects with a history of psychiatric disorders, current substance abuse, head injuries with concomitant loss of consciousness, seizures, central nervous system infection, diabetes, or hypertension were excluded. All subjects provided informed written consent before participation in this study. The institutional review board of the National Institute of Mental Health reviewed and approved this study.

NEUROPSYCHOLOGICAL TESTS

Neuropsychological tests assessing several cognitive domains were administered to all subjects for 1 to 3 sessions by a practicing psychologist or psychometrician (T.W.W., T.E.G., J.M.G.) trained in administration and scoring of all tests. Scoring followed standardized procedures. Logical Memory I and II and Visual Reproduction I and II of the Wechsler Memory Scale–Revised (WMS-R)[24] and the California Verbal Learning Test (CVLT)[25] were administered as tests of declarative memory. The Boston Naming Test (BNT)[26] and Word Fluency[27] were administered as tests of verbal retrieval and lexical integrity. The vigilance and distractibility versions of the Gordon Continuous Performance Test (CPT)[28] were administered as tests of attention processes. A 128-card version of the Wisconsin Card Sorting Test (WCST)[29] was administered as a test of executive function and set shifting. The Benton Line Orientation Test[30] was administered as a test of visuospatial perceptual abilities. Forms A and B of the Trail-Making Test[31] were administered as tests of psychomotor speed. The Finger Tapping Test[32] was administered as a test of motor speed.

Current FSIQ Estimate

All subjects were administered a 4-subtest version of the WAIS-R,[33] consisting of the Arithmetic, Digit Symbol Substitution, Picture Completion, and Similarities subtests, to obtain an estimate of their current FSIQ.[34,35]

Premorbid IQ Based on WRAT-R Reading Scores

All subjects received the Reading subtest of the WRAT-R[36] to obtain an estimate of premorbid intellectual levels. The Reading subtest of the WRAT-R is thought to reflect preserved abilities, since it is a test of decoding skills that are routinely acquired before the onset of disease and appear to remain unaffected by the disease process in analogous fashion to the hold subtests (those tests that are insensitive to deterioration associated with normal aging and certain types of brain damage[37]) of the WAIS-R.[3,38,39] In monozygotic twin pairs discordant for schizophrenia, the unaffected twin scored on average 10 points higher than the affected twin with respect to IQ, whereas WRAT-R Reading scores were equivalent.[4] Furthermore, previous

of intellectually declining patients would display executive function, attention, and memory deficits; a nondeclining, high-functioning group would display a milder and more restricted range of deficits; and a group with both premorbid and morbid IQ deficits would display a broader spectrum of cognitive impairment. Healthy control subjects were included as a comparison group to determine the degree to which patients with schizophrenia deviate from normal on different cognitive measures and as a means of providing a form of validity for the use of Reading scores as a premorbid intellectual measure.

To validate our patient grouping strategy, atheoretical cluster analyses were performed using Wechsler Adult Intelligence Scale–Revised (WAIS-R) Full-Scale Intelligence Quotient (FSIQ) and WRAT-R Reading scores. Cluster weights and between-cluster analyses of variance (ANOVAs) provided indices of internal homogeneity and external validity. Canonical analyses comparing IQ and FSIQ–WRAT-R Reading difference scores with all other cognitive measures were performed to determine the extent to which IQ and difference scores account for unique, nonredundant variance.

RESULTS

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Table 2 lists sex ratios, mean age, duration of illness, age of first symptom onset, and age of first hospitalization. Duration of illness was measured from the time of each patient's first hospitalization.

At the time of testing, most patients (87%) were receiving typical neuroleptic medications, usually haloperidol, fluphenazine hydrochloride, molindone hydrochloride, or thioridazine hydrochloride. The remaining patients (13%) were receiving atypical neuroleptic medications, either clozapine or risperidone. Eighty-six percent of the preserved group, 83% of the compromised group, and 92% of the deteriorated group were receiving typical neuroleptic medication. No cognitive differences on the basis of medication status were discerned among the groups.

CLINICALLY BASED SUBGROUPING ANALYSES

Intellectual decline of at least 10 points from premorbid levels as measured by WRAT-R Reading occurred in ap-

studies have consistently demonstrated reading scores to be viable measures of premorbid intellect.[38-42]

Support for the validity of using WRAT-R Reading standard scores as measures of general intellect in the normal population can be found in **Table 1**, which demonstrates nearly identical means for WRAT-R Reading standard scores and the 4-subtest version of the WAIS-R FSIQ in the healthy control group. Consistent with other healthy samples,[43] the WAIS-R FSIQ and WRAT-R Reading standard scores in our healthy group were significantly correlated ($r=0.74$, $P<.001$). The SD of the FSIQ–WRAT-R Reading difference scores was 11.5 in the total sample.

CLASSIFICATION OF PATIENTS

Clinically Derived Groups

Based on previous findings that demonstrated high-functioning, deteriorated, and compromised patients with schizophrenia,[1,4,8,23,44,45] patients were classified into 1 of 3 intellectual groups: (1) those displaying a meaningful decline in IQ (≥10 points) as evidenced by the difference between current IQ (based on a 4-subtest version of the WAIS-R FSIQ) and premorbid IQ (based on WRAT-R Reading standard score), hereafter referred to as *intellectually deteriorated*; (2) those displaying premorbid IQ based on WRAT-R Reading scores that were below 90, hereafter referred to as *intellectually compromised*, which is consistent with the work of David et al[18] and with conventional usage (less than the 16th percentile)[33]; and (3) those whose premorbid IQs based on WRAT-R Reading scores were above 90 and who demonstrated less than a 10-point difference between their premorbid IQ based on WRAT-R Reading and their current IQ, hereafter referred to as *intellectually preserved*. Existence of a 10-point IQ decline took precedence to either of the cutoff strategies described.

Empirically Derived Groups

Atheoretical canonical and cluster analyses were applied to the data to determine the validity of our clinically derived patient grouping strategy. We performed a cluster analysis on FSIQ and WRAT-R Reading scores using complete linkage and Squared Euclidean Distances to determine the number of clusters that might be present in the patient sample. Examination of the resulting dendrogram, a tree diagram that displays how individual observations are grouped, suggested a 4-cluster solution would be appropriate. Next we entered the data into a k-means cluster analysis, with the number of clusters equal to 4. Uniformly high classification accuracy across clusters (77.8% for cluster 1, 100% for cluster 2, 93.8% for cluster 3, and 100% for cluster 4) demonstrates excellent separation of the clusters. It is important to emphasize that the algorithm defining these groups was based entirely on FSIQ and WRAT-R Reading scores.

STATISTICAL ANALYSES

A χ^2 analysis was used to evaluate the dichotomous variable of diagnostic subtype in relation to intellectual subgroup. A χ^2 partitioning procedure was used to determine which of the diagnostic subtypes were represented among the intellectual subgroups beyond expected values. The χ^2 partitioning procedure allows more detailed analysis of a contingency table for which a significant χ^2 value has been obtained.[46]

With respect to the parametric data collected from the various neuropsychological tests, a series of ANOVAs were performed to determine which variables differed significantly among the different intellectual subgroups and healthy controls. For each of the ANOVAs, results were considered to be significant after consistently and uniformly applying a Bonferroni correction for multiple comparisons using $P\leq.002$, unless otherwise noted. Predetermined post hoc contrasts using least significant difference (LSD) analyses (with α set at .05) were performed on the basis of hypothesized differences among groups. All statistical analyses were based on 2-tailed tests of significance.

proximately half (51.3%) of the patients (the intellectually deteriorated group). Nearly a quarter of the patients (23.9%) showed low premorbid intellect based on WRAT-R Reading scores, combined with low average current IQ (the intellectually compromised group). The remaining 24.8% of the patients were intellectually preserved with both current and premorbid IQ based on WRAT-R Reading scores within normal limits. Table 1 provides the mean IQ and WRAT-R Reading standard scores for patients and controls.

NEUROPSYCHOLOGICAL DEFICITS

There were significant differences among groups on immediate and delayed visual reproduction from the WMS-R, the number of items recalled correctly from list A (trials 1-5) and the number of items recalled correctly during free recall after short and long delays from the CVLT, the number of items correctly named on the BNT, the number of items correctly identified on the Benton Line Orientation Test, a composite of the number of correct responses obtained during both the vigilance and distractibility portions of the CPT, the percentage of perseverative errors and the number of categories attained on the WCST, and the mean time to complete forms A and B of the Trail-Making Test (**Table 3**). After applying a Bonferroni correction, immediate and delayed logical memory of the WMS-R displayed near-significant differences among groups (Table 3). There were no significant differences among groups with respect to word fluency and finger tapping (Table 3). Post hoc LSD testing showed that the compromised group differed significantly from the preserved and healthy control groups on immediate and delayed visual reproduction of the WMS-R, all the measures of the CVLT, the BNT, the Benton Line Orientation Test, the CPT, the WCST, and Trails A and B (Table 3). Further LSD follow-up analyses revealed that the deteriorated group differed significantly from the preserved and healthy control groups on immediate and delayed visual reproduction of the WMS-R, all the measures of the CVLT, the WCST, and Trails A and B (Table 3). Additionally, the deteriorated group differed significantly from the healthy control group on the CPT and from the preserved group on line orientation (Table 3). Additional post hoc LSD analy-

Table 1. Mean (SD) Scores on Tests of Intellectual Abilities for Patients With Schizophrenia and Healthy Controls*

	Healthy Controls (n = 21-26)	Patients With Schizophrenia			F	df	P	Post Hoc Analysis†
		Preserved (n = 29)	Deteriorated (n = 56-60)	Compromised (n = 28)				
Wechsler Adult Intelligence Scale–Revised								
Arithmetic	10.27 (3.07)	10.52 (2.80)	7.22 (2.53)	5.93 (1.72)	23.89	139	<.001	a
Similarities	10.72 (2.54)	11.90 (2.35)	9.18 (2.88)	8.18 (1.54)	13.03	138	<.001	a
Picture Completion	9.77 (3.29)	9.93 (2.12)	8.21 (2.19)	7.75 (2.19)	6.44	139	<.001	a
Digital Symbol Substitution	9.52 (3.04)	8.21 (2.38)	6.29 (1.91)	6.12 (1.93)	15.19	133	<.001	b
Full-scale IQ	101.32 (13.58)	101.97 (11.50)	87.77 (10.13)	80.86 (5.11)	29.64	138	<.001	a
Wide Range Achievement Test–Revised								
Reading standard score	101.39 (16.20)	100.62 (11.62)	104.87 (10.51)	81.29 (9.47)	27.08	136	<.001	c

*Sample size varied with patient compliance and ability.
†a indicates healthy controls significantly different from preserved and deteriorated patients; b, healthy controls significantly different from all patient groups; and c, healthy controls significantly different from compromised patients.

Table 2. Demographic Characteristics*

Characteristic	Healthy Controls (n = 27)	Patients With Schizophrenia		
		Preserved (n = 29)	Deteriorated (n = 60)	Compromised (n = 28)
Age, mean (SD), y	26.7 (9.9)	34.9 (6.8)	33.7 (9.1)	32.1 (8.1)
M/F	15/12	21/8	44/16	19/9
Duration of illness, mean (SD), y	...	11.9 (7.3)	12.4 (7.8)	9.4 (8.7)
Age of symptom onset, mean (SD), y	...	20.5 (4.8)	18.5 (4.9)	19.3 (4.6)
Age of first hospitalization, mean (SD), y	...	23.8 (5.9)	22.0 (6.1)	22.3 (5.4)
Diagnostic subtype, No. (%)				
Undifferentiated	...	18 (15.7)	34 (29.6)	22 (19.1)
Paranoid	...	10 (8.7)	14 (12.2)	6 (5.2)
Disorganized	...	1 (0.9)	10 (8.7)†	0 (0.0)

*The residual subtype was omitted because of the low incidence of patients in that category; therefore, the total number of patients with schizophrenia in this analysis equals 115.
†Significant deviation from expected frequency based on a χ^2 partitioning procedure where $P<.01$.

ses demonstrated that the compromised group was significantly different from the deteriorated group on the basis of the BNT, Benton Line Orientation Test, and the number of categories attained on the WCST (Table 3). Finally, post hoc LSD analyses revealed that the preserved group differed significantly from the healthy control group on the basis of the CPT and number of categories attained on the WCST (Table 3).

OTHER RELEVANT VARIABLES

Based on a series of 1-way ANOVAs, there were no significant differences among any of the patient groups with respect to the duration of illness, age of first symptom onset, or age of first hospitalization (Table 2). The difference between the age of first symptom onset and the age of first hospitalization ranged from 3.0 to 3.5 years for each of the 3 groups.

INTELLECTUAL SUBGROUP AND DIAGNOSTIC SUBTYPE

On the basis of a χ^2 analysis, diagnosis was found to be significantly associated with the intellectual subgroups ($n=115$, $\chi^2_4=9.69$, $P=.05$). Disorganization was more likely to occur in the intellectually deteriorated group ($n=85$, $\chi^2_1=7.75$, $P=.01$) (Table 2). There were no other deviations from expected frequencies with respect to the occurrence of a specific diagnostic subtype of schizophrenia in any of the neuropsychological subgroups.

EMPIRICALLY BASED SUBGROUP ANALYSES

Canonical Correlation Analyses

A separate canonical correlation procedure applied to the total sample of patients with schizophrenia revealed that root 1, on which FSIQ loaded primarily (−1.00) and the WAIS-R minus WRAT-R Reading difference score loading minimally (0.05), accounted for 0.20 variance ($n=117$, $\chi^2_{42}=168.28$, $P<.001$). Root 2, on which the difference score loaded (1.00) (FSIQ loaded minimally, 0.007), accounted for 0.06 variance ($n=117$, $\chi^2_{20}=38.91$, $P=.007$). These results indicate that both FSIQ and the FSIQ-WRAT-R Reading difference scores make independent contributions to the variance in other cognitive domains.

Table 3. Mean (SD) Scores on Neuropsychological Tests for Patients With Schizophrenia and Healthy Controls*

	Healthy Controls (n = 19-24)	Patients With Schizophrenia			F	df	P	Post Hoc Analysis†
		Preserved (n = 20-29)	Deteriorated (n = 45-60)	Compromised (n = 21-28)				
Wisconsin Card Sorting Test								
Categories	8.00 (2.50)	5.84 (2.48)	4.23 (3.31)	2.78 (2.24)	15.84	3, 116	<.001	f
Perseverative errors, %	11.34 (6.89)	16.05 (9.94)	22.36 (15.42)	26.65 (13.60)	6.40	3, 112	<.001	a
Gordon Continuous Performance Test								
Vigilance and distractibility total correct	56.84 (4.40)	51.04 (11.28)	47.62 (8.96)	45.57 (8.99)	6.61	3, 111	<.001	c, e
Benton Line Orientation Test	24.24 (4.49)	26.72 (2.95)	23.60 (5.15)	21.04 (5.52)	6.80	3, 131	<.001	d, e, g
Trail-Making Test								
Form A	25.50 (8.10)	36.14 (10.37)	49.31 (25.04)	46.57 (19.80)	9.32	3, 134	<.001	a
Form B	59.73 (18.80)	75.21 (27.24)	135.54 (78.04)	136.64 (75.66)	12.41	3, 134	<.001	a
Finger Tapping Test								
Dominant hand	48.42 (6.03)	48.92 (7.13)	45.47 (7.44)	45.23 (10.32)	1.70	3, 123	.17	h
Nondominant hand	45.42 (5.57)	44.26 (6.56)	41.00 (6.62)	42.74 (14.36)	1.63	3, 124	.18	h
California Verbal Learning Test								
List A trials 1-5	54.35 (11.52)	49.77 (8.34)	37.64 (13.61)	38.62 (11.25)	13.29	3, 107	<.001	a
Short-delay free recall	10.78 (3.40)	10.45 (2.48)	7.51 (3.52)	7.38 (3.34)	8.02	3, 105	<.001	a
Long-delay free recall	11.57 (2.83)	10.75 (2.63)	7.73 (3.73)	7.67 (3.07)	10.16	3, 105	<.001	a
Word Fluency	43.70 (10.58)	38.66 (11.91)	36.63 (10.86)	34.52 (9.32)	3.43	3, 135	.009	h
Boston Naming Test	51.52 (6.52)	53.41 (3.75)	50.69 (7.11)	45.85 (6.50)	7.13	3, 131	<.001	e
Wechsler Memory Scale–Revised								
Logical Memory I	26.88 (6.71)	23.24 (6.93)	18.68 (9.31)	17.86 (7.27)	4.50	3, 120	.005	h
Logical Memory II	23.63 (6.93)	19.07 (7.17)	13.93 (9.84)	14.07 (8.44)	4.67	3, 119	.004	h
Visual Reproduction I	35.37 (3.22)	33.34 (5.81)	27.56 (9.74)	27.86 (7.37)	7.28	3, 131	<.001	a
Visual Reproduction II	29.68 (8.51)	28.97 (7.83)	21.33 (12.00)	20.50 (7.79)	7.02	3, 130	<.001	a

*Sample size varied with patient compliance and ability.

†a indicates healthy controls and preserved patients significantly different from compromised and deteriorated patients; c, healthy controls significantly different from all patient groups; d, preserved patients significantly different from compromised and deteriorated patients; e, compromised patients significantly different from all other groups; f, all groups significantly different from each other; g, deteriorated patients significantly different from all other patient groups; and h, no significant differences among groups.

Cluster Analyses

Cluster 1 was composed of 9 patients displaying high FSIQ and WRAT-R Reading scores. This atheoretically derived group was similar in nature to our previously defined preserved group. Cluster 2 was composed of 52 patients displaying a mean 8.9-point intellectual decline based on WRAT-R Reading score. Cluster 3 was composed of 32 patients displaying a mean 14.9-point intellectual decline based on WRAT-R Reading score. Taken together, these 2 declining clusters are analogous to our deteriorated group. In the fourth cluster, consisting of 24 patients, both FSIQ and WRAT-R Reading scores were well below average, consistent with our previously described compromised group.

We next used a series of ANOVAs to examine between-cluster differences on all non-FSIQ and WRAT-R Reading cognitive variables to assess within-cluster homogeneity and between-cluster heterogeneity (data available on request). The patterns of cognitive impairment among the atheoretical clusters were analogous to the patterns obtained on the basis of clinically driven cutoff scores. Thus, cluster 4 differed from all other groups on the WCST, CPT, memory tests, and, importantly (analogous to our previously described results in Table 3), tests of language and visual processing (line orientation and naming). In contrast, the 2 declining clusters exhibited impairments on CPT, memory, and WCST vis-à-vis healthy controls but no deficits on naming or line orientation. The preserved cluster did not differ from the healthy control group on any of the cognitive variables assessed.

Parenthetically, however, we note that the mean FSIQ score was higher in cluster 1 (mean, 116.11) than in the healthy control group (mean, 101.32). Therefore, a subsequent ANOVA was performed in which the patients with schizophrenia from cluster 1 (FSIQ: mean, 116.11; SD, 11.13) were matched on the basis of WAIS-R FSIQ score with subjects from the healthy control group (FSIQ: mean, 112.44; SD, 7.20). The patients with schizophrenia from cluster 1 differed significantly from this matched healthy control group on the number of categories obtained in the WCST (patients from cluster 1: mean number of categories, 6.78; SD, 2.77; matched controls: mean number of categories, 9.44; SD, 0.88; $F_{1,16}=7.55$, $P<.01$). This result is consistent with the result obtained via the clinically driven cutoff scores. An ANOVA on the IQ-matched groups for CPT distractibility correct scores was not significant (cluster 1: mean number correct, 28.44; SD, 2.13; matched controls: mean number correct, 28.71; SD, 2.36).

COMMENT

In a large sample of patients consecutively admitted to a tertiary referral center, we found evidence for distinct patterns of cognitive dysfunction in schizophrenia. The results support previous findings of intellectual decline based on WRAT-R Reading scores in schizophrenia with associated deficits of attention, memory, executive function,

and oculomotor speed, although this diminution was obtained in only half of the inpatients with chronic schizophrenia in this sample. The remaining 50% did not appear to experience a significant intellectual decline. Of these nondeclining patients, approximately half (ie, 25% of the total sample) appear to be compromised on the basis of displaying mildly impaired premorbid IQs based on WRAT-R Reading scores and impairment in a wide variety of cognitive domains. The remaining patients evince a neuropsychological profile that resembles normal, with the selective exception of specific executive function and possibly attention deficits. The argument may be made that the delineation of IQ patterns creates a tautology, since all patients necessarily had to meet criteria for one category or another. However, there was no a priori reason to believe that the distribution would be as it was or that the preserved or compromised groups would display the pattern of deficits observed.

To validate our clinically driven subgroup strategy, we applied cluster and canonical correlation analyses to the patient data set. Cluster analytical results were consistent with the clinically driven cutoff score results. The clusters differed significantly from one another and from healthy controls in a manner that was broadly and strikingly similar to the patterns observed using clinically driven subgroups, thus providing a measure of external validity. Canonical correlation analysis demonstrated that IQ and IQ minus WRAT-R Reading score independently predicted other cognitive measures, providing weight to the fulcrum of our study. Although previous work[22,47] has demonstrated heterogeneity in the cognitive deficits displayed in schizophrenia, the present findings extend their results by demonstrating preservation and impairment for a wide variety of cognitive domains that follow in a principled way from patterns of preserved and compromised intellect.

The pattern of memory, visuospatial perception, attention, executive function, language, and psychomotor deficits in the compromised group was similar to the finding of Russell et al,[8] who found low premorbid IQ (mean IQ, 84.1) in a sample of children who had early contact with child guidance clinics. These findings implicate widespread cortex dysfunction in the compromised group. The modal deteriorated patient group in our sample displayed an intellectual decline based on the use of WRAT-R Reading as a measure of premorbid intellect.[4-6,38,39,44,48,49] We recognize that this intellectual decline does not affect all cognitive domains equally. Intellectual decline observed in the deteriorated patient group was accompanied by memory, executive function, attention, psychomotor speed, and oculomotor scanning impairments and implicates frontotemporal dysfunction.[50] The pattern of cognitive deficits in deteriorated patients does not preclude a neurodevelopmental mechanism in the etiology of cognitive deficits in schizophrenia, since subtle neurodevelopmental changes may precede and set the stage for later cognitive impairment and psychiatric disturbance.[51] Furthermore, previous studies have demonstrated that this intellectual decline is limited to the period around symptom onset rather than being progressive throughout the illness.[52,53]

An unexpectedly large minority of patients (about 25%) were intellectually intact. This group may be informative in several respects. First, they confirm that antipsychotic drug therapy does not necessarily compromise performance on numerous cognitive tests. Second, they demonstrate that chronic schizophrenia can exist in the context of preserved intellect and cognition. This group may also speak to the ongoing controversies about whether there is a core cognitive deficit associated with schizophrenia. These patients displayed mild impairment only in the cognitive domain of executive function and, possibly, attention and encoding.

The preserved group was generally similar to those intellectually preserved groups previously described.[20-23,45,54] However, these cognitively intact patients appeared to be subtly impaired on the WCST relative to controls displaying equivalent overall ability, consistent with the results of Elliott et al,[45] who observed impaired performance on an analogue of the WCST in patients who displayed otherwise intact intellect.

There are several limitations to the present study. First, the design of this study was not longitudinal, and we did not directly obtain premorbid IQ scores. Clearly, using actual premorbid IQ estimates would make the strongest argument for intellectual decline with the onset of schizophrenia, and our results would suggest that such a study is warranted. A second limitation refers to the representativeness of our sample. Although we routinely admit patients with chronic disease, we believe that our sample is representative since we observed high-functioning patients, our total patient mean FSIQ of about 90 is similar to others, and our sex ratios are consistent with others.[55-57] Although an imperfect overlap between our atheoretical clusters and clinically driven subgroups constitutes a third limitation, we were struck by the fact that the atheoretical procedure would generate homogeneous groups, approximating real-world phenomena.

It is also possible that our results might be driven by general intelligence. Results of a principal components analysis of our patient data militate against this possibility. Briefly, 3 factors were extracted. The first might be considered a prefrontal executive or attention factor (with WCST loading), the second can be considered a verbal memory factor (with WMS-R logical memory loading), and the third can be considered to reflect IQ (with WAIS-R, WRAT-R Reading, language, and visual spatial processing loading). (Results are available on request.) Thus, IQ does not fully predict other cognitive impairments.

Cognitive deficits associated with schizophrenia, including those in intelligence, may emerge along several hypothetical developmental trajectories. One course may be characterized by profound and widespread cognitive impairment manifest from early development prior to psychotic symptom onset. A second course may be characterized by a circumscribed deficit pattern that includes intellectual decline and encompasses the domains of executive function, attention, and episodic memory, and may approximately coincide with psychotic symptom onset. Finally, a third group of patients have subtle cognitive deficits, apparently restricted to the domain of executive function. It is unclear whether these deficits precede or coincide with the onset of clinical symptoms.

It would appear that deficits associated with the function of the prefrontal cortex, ie, executive function defi-

cits (as indexed by the number of categories attained on the WCST), constitute a necessary type of cognitive impairment in schizophrenia, given their presence in the intellectually compromised and preserved groups, and are in keeping with the findings of Shallice et al,[58] who also found consistent evidence for executive impairment. Results from this study also appear to synthesize the schizophrenia neuropsychological literature with respect to (1) intellectual decline based on WRAT-R Reading score, (2) the presence of premorbid deficits based on WRAT-R Reading score, (3) a modal cognitive deficit pattern, and (4) preserved cognitive abilities. Previous studies have generally tended to focus on only 1 of the 3 cognitive groups of patients with schizophrenia.

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Correction

Error in Table Footnote. In the original article by Weickert et al titled "Cognitive Impairments in Patients With Schizophrenia Displaying Preserved and Compromised Intellect," published in the September 2000 issue (2000;57:907-913), Table 1 on page 910, the first part of the second footnote should have read "a indicates healthy controls significantly different from deteriorated and compromised patients; . . . "

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Distribution and localization of pro-brain-derived neurotrophic factor-like immunoreactivity in the peripheral and central nervous system of the adult rat

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Abstract

The precursors for neurotrophins are proteolytically cleaved to form biologically active mature molecules which activate their receptors p75NTR and trks. A recent study showed that the precursor for nerve growth factor (NGF) can bind to p75NTR with a high affinity and induces apoptosis of neurons *in vitro*. Mutation in Val66Met of brain-derived neurotrophic factor (BDNF) results in reduction in hippocampal function in learning and in the dysfunction of intracellular BDNF sorting and secretion. To examine the functions of pro-neurotrophins *in vivo*, it is essential to know where they are expressed in the nervous system. In the present study, we have raised and characterized rabbit polyclonal antibodies against a peptide coding for the precursor region of the BDNF gene. The antibody specifically recognizes the precursor for BDNF by western blot. With the affinity purified precursor antibody, we have mapped the distribution and localization of the precursor for BDNF. The results showed that, like mature BDNF, pro-BDNF is localized to nerve terminals in the superficial layers of dorsal horn, trigeminal nuclei, nuclei tractus solitarius, amygdaloid complex, hippocampus, hypothalamus and some peripheral tissues. These results suggest that pro-BDNF, like mature BDNF, is anterogradely transported to nerve terminals and may have important functions in synaptic transmission in the spinal cord and brain.

Keywords: immunohistochemistry, neurotrophins, proBDNF.
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Neurotrophins are a family of structurally similar proteins which play important roles in proliferation, differentiation and survival of neurons in the peripheral and central nervous systems (PNS and CNS) during development (Davies 1994). The gene products of neurotrophins after synthesis are around 35 kDa proteins which are proteolytically cleaved to form biologically active mature molecules (13.5 kDa) by removal of the preproregions within cells (Kolbeck *et al.* 1994; Seidah *et al.* 1996; Mowla *et al.* 1999; Farhadi *et al.* 2000). The mature molecules form homodimers, are secreted from cells or nerve terminals through constitutive or regulatory pathways (Heymach *et al.* 1996; Balkowiec and Katz 2002) and activate their receptors, p75NTR and trks (Barker and Murphy 1992). In addition to their roles in developing neurons, neurotrophins also play critical roles in the synaptic activity and plasticity in many groups of mature neurons. Of particular interest is brain-derived neurotrophic factor (BDNF) which is widely expressed in the CNS and PNS (Yan *et al.* 1997), is anterogradely transported after synthesis (Zhou and Rush 1996b), stored in the nerve terminals (Luo *et al.* 2001), released in an activity-dependent manner and participates in synaptic plasticity (Poo 2001; Lu 2003a).

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Abbreviations used: Arc, arcuate hypothalamic nucleus; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CVLM, ventrolateral medulla; d.g., dentate gyrus; DRG, dorsal root ganglia; ECL, enhanced chemo-luminescent; GFP, green fluorescence protein; IML, intermediolateral column; i.p., intraperironeal; KLH, keyhole limpet hemocyanin; ME, medial eminence; NGF, nerve growth factor; NTS, nuclei tractus solitarius; PBS, phosphate-buffered saline; PNS, peripheral nervous system; Pro-BDNF, pro-brain derived neurotrophic factor; ProNGF, Pro-nerve growth factor; SDS, sodium dodecyl sulfate; Spt5, spinal tract of trigeminal nerve; VMH, ventromedial hypothalamic nuclei.

Recent studies suggest that neurotrophin precursors and the enzymatically cleaved pro-region fragments may also have important physiological functions. For example, the precursor for nerve growth factor (NGF) is released from cells and the unprocessed full-length precursor can bind to p75NTR with high affinity and induce apoptosis of neurons and glia (Lee *et al.* 2001; Beattie *et al.* 2002). In another study, Egan *et al.* have shown that a single amino acid mutation (Val66Met) in the proregion of BDNF affects the sorting of the molecule into the nerve terminals, retards activity-dependent secretion and reduces learning functions of the hippocampus in humans (Egan *et al.* 2003; Hariri *et al.* 2003). Most recently, proNGF was shown to bind a third receptor, sortilin, with a high affinity, form a complex with p75NTR and cause apoptosis (Nykjaer *et al.* 2004). It is possible that proneurotrophins are not merely precursors and may play an important role in the physiological and pathological functions of nervous system.

Despite evidence indicating proneurotrophins are dominant gene products in the normal human brain and the brains of Alzheimer's and Parkinson's disease patients (Fahnestock *et al.* 2001; Fahnestock *et al.* 2002; Michalski and Fahnestock 2003), it is not known where proneurotrophins are localized. To understand the functions of proneurotrophins *in vivo*, knowledge of both localization and distribution is essential. This requires an antibody that is specific for proneurotrophins and does not recognize the mature molecule. As BDNF is widely distributed in the nervous system and expressed in the brains of adult animals, we have raised and characterized rabbit polyclonal antibodies against a peptide coding for the precursor region of the BDNF gene. We have examined the localization and distribution of proBDNF in the rat PNS and CNS by an immunohistochemical method. We found that proBDNF is localized predominantly in nerve terminals *in vivo* and shares a similar distribution pattern to the mature molecule in rats.

Materials and methods

Animals

All procedures involving animals were approved by the Animal Welfare Committee of Flinders University and undertaken according to the guidelines of the National Health and Medical Research Council of Australia. All animals were kept under standardized barrier-breeding conditions (12-h light/12-h dark cycle) with free access to water and food. Male and female Sprague–Dawley rats weighing 250–350 g were used.

Antibody production

The antibody was raised in rabbits against peptide sequence 69–82 of the human BDNF gene, which was conjugated with keyhole limpet hemocyanin (KLH) for immunization. Two adult New Zealand rabbits were used for immunization. The antibodies were affinity purified against the immunizing peptide which was immobilized on Sepharose 4B gel.

Western blot

Rats were anaesthetized Nembutal [80 mg/kg, intraperironeal (i.p.)] and killed by decapitation. Fresh dorsal root ganglia (DRG), spinal cord, pituitary gland and hypothalamus tissues were homogenized in 10 mM Tris buffer (pH 7.4) with protease inhibitors (one tablet in 50 mL, Roche Diagnostic, Switzerland) at a weight/volume ratio of 1 mg/10 μL. The samples were centrifuged at 10 000 *g* for 15 min and the supernatant collected for western blot. Protein concentrations in the samples were assayed using Bradford reagents (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. A total 32 μg protein from each tissue sample and 1 ng of recombinant mouse proBDNF were loaded onto the 12% sodium dodecyl sulfate (SDS) gel, separated by electrophoresis and transferred to nitrocellulose membrane. Recombinant mouse proBDNF was made in *Escherichia coli* using pENTR directional TOPO cloning kits (Invitrogen), according to the manufacturer's instructions. The membranes were probed by affinity purified proBDNF antibodies (0.5 μg/mL) overnight followed with HRP-labeled goat anti-rabbit IgG (Sigma, 1 : 5000) for 1 h. The specific bands were detected by the enhanced chemoluminescent (ECL) method.

To examine the specificity of the western blot, 50 ng recombinant proBDNF and spinal cord extract (32 μg protein) were loaded on duplicate gels. After electrophoresis and protein transfer onto membranes, one membrane was probed with affinity-purified rabbit proBDNF antibodies as described above and the other was probed with the same antibodies which were preabsorbed in the presence of the peptide (10 μg/mL).

Immunohistochemistry of proBDNF

Adult male and female rats were anaesthetized with Nembutal (80 mg/kg, i.p.) and perfused with 1% sodium nitrite in phosphate buffered saline (PBS) briefly followed with 300 mL 4% paraformaldehyde. Brain, ganglia and peripheral tissues were dissected and postfixed in 4% paraformaldehyde overnight. After brief rinsing in PBS, tissues were soaked in 30% sucrose overnight. All tissues were cut into 40-μm sections on a cryostat (Leica). Freely floating sections were washed briefly in PBS and incubated in 50% ethanol containing 1% hydrogen peroxide for 30 min. After thorough washing in PBS, sections were blocked in 20% normal horse serum for 2 h and incubated in either preimmune serum or affinity purified antibodies to proBDNF (0.5 μg/mL) overnight at room temperature. Following sequential incubations in the biotin-labelled secondary antibodies and the ABC kit, the sections were developed in a solution of diaminobenzadine and hydrogen peroxide with nickel enhancement. Some sections were incubated with antibodies preabsorbed with immunizing peptide (at 10 μg/mL) or recombinant proBDNF (10 μg/mL) or recombinant human BDNF (10 μg/mL) to examine the specificity of the antibodies for immunostaining. The labeling intensity and density of positive cells and fibers were jointly assessed on each region and scored on a arbitrary scale of –, +, + +, and + + +, representing no labeling, weak, moderate and strong labeling, respectively.

Transfection of PC12 cells with proBDNF plasmids

To further confirm the specificity of the antibody, NGF-induced differentiated PC12 cells were used for transfection experiments (Kojima *et al.* 2001). Briefly, PC12 cells were cultured in six-well culture plates (2×10^3cells/well) in DMEM medium containing 10% normal horse serum, 5% fetal calf serum and 0.5 mM L-glutamine. After removal of the serum, the cells were treated with β-NGF (100 ng/mL) or conditioned medium for 2 days. Then the cells were transfected with plasmid pro-BDNF-EGFP (1.25 μg/10^3 cells) (Kojima *et al.* 2001) or plasmid EGFP (1.125 μg/10^3 cells) by the FuGENE™ 6 transfection reagent (Boehringer Mannheim Corp). Three days after transfection, the cells were fixed in 4% paraformaldehyde and pro-BDNF immunohistochemistry was performed as described above. For an additional positive control, adult rat DRG were dissected and dissociated by incubation in collagenase (5000 U/mL, type IV, Sigma) and trypsin-EDTA (Gibco). Dissociated DRG cells were cultured for 3 days on coverslips coated with laminin/poly L-lysine before staining for proBDNF as described above.

Results

Western blot

Western blot analysis was used to examine exogenous recombinant proBDNF and the expression of endogenous proBDNF in several tissues. As shown in Fig. 1(a), the rabbit polyclonal antibodies recognized recombinant proBDNF made in *E. coli* and a major band corresponding to proBDNF was present in the spinal cord, DRG, pituitary gland and hypothalamus, the areas with strong immunoreactivity to proBDNF. Two bands were detected in pituitary gland, possibly corresponding to the differential processing or glycosylation as demonstrated in previous studies (Mowla *et al.* 2001). To demonstrate the specificity of the band in western blot, duplicate membranes, on which a large amount (50 ng) of exogenous recombinant proBDNF and spinal cord tissue extract were loaded, were probed by the antibodies in the absence or presence of the immunizing peptide. As shown in Fig. 1(b), in the absence of immunizing peptide, the antibody detected a heavy band of exogenous proBDNF and a weak band of endogenous proBDNF at 35 kDa in the spinal cord homogenate (lane 2, Fig. 1b). In contrast, in the presence of the immunizing peptide, all bands were completely abolished in this parallel experiment (Fig. 1c).

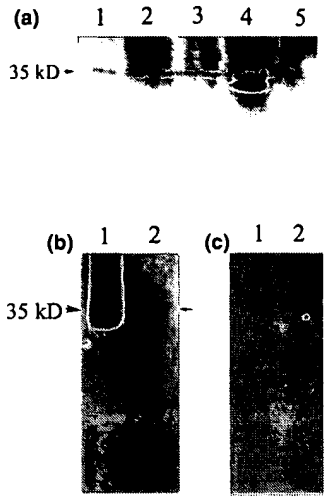

Fig. 1 Characterization of the rabbit polyclonal antibodies to proBDNF by western blots. (a) Western blot analysis of recombinant and endogenous proBDNF. Lane 1, 1 ng of recombinant proBDNF made in *E. coli*; lane 2, spinal cord; lane 3, DRG; lane 4, pituitary gland; lane 5, hypothalamus. (b) Western blot of a larger amount of proBDNF (50 ng, lane 1) and spinal cord extract (lane 2) probed by the rabbit proBDNF antibodies in the absence of the immunizing peptide. Large arrow indicates a heavy band of exogenous ProBDNF and the small arrow indicates a light band of endogenous BDNF present in the spinal cord extract. (c) A parallel western blot with the same conditions as in (b) but the membrane was probed with the antibodies in the presence of 10 μg/mL immunizing peptide. All bands were completely abolished by the peptide.

In vitro transfection and culture of DRG neurons

To see whether the antibodies were useful for immunolocalization studies as proposed, we tested PC12 cells transfected with the proBDNF plasmid and cultured DRG cells which are known to express high levels of BDNF (Acheson *et al.* 1995). About 10% of proBDNF positive cells were stained in proBDNF-transfected PC12 cells (Fig. 2b) but not in control plasmid transformed cells (Fig. 2a). A subpopulation of cultured DRG neurons were intensely labeled (arrows, Fig. 2c). Having established the specificity of the antibody for proBDNF, the next major part of the study was to use this antibody as a tool to determine the localization and distribution of proBDNF in both PNS and CNS.

Immunohistochemistry controls

No staining was seen if the sections were incubated in the preimmune serum (data not shown). The staining described in this study was entirely absorbed in the presence of 10 μg/mL of the immunizing peptide (see below on the result of the spinal cord and medulla). The staining was also dramatically reduced when the sections were incubated with the antibodies preabsorbed with recombinant proBDNF (see below), further indicating the specificity of staining. Similar results were obtained in other regions such as hippocampus, hypothalamus, amygdala and pituitary gland. However, the specific immunostaining in the dorsal horn of the spinal cord (Fig. 3a) and other brain regions was not affected by the preabsorption with a high concentration of mature BDNF (10 μg/mL) (Fig. 3b), suggesting the specificity for the proregion of the gene product.

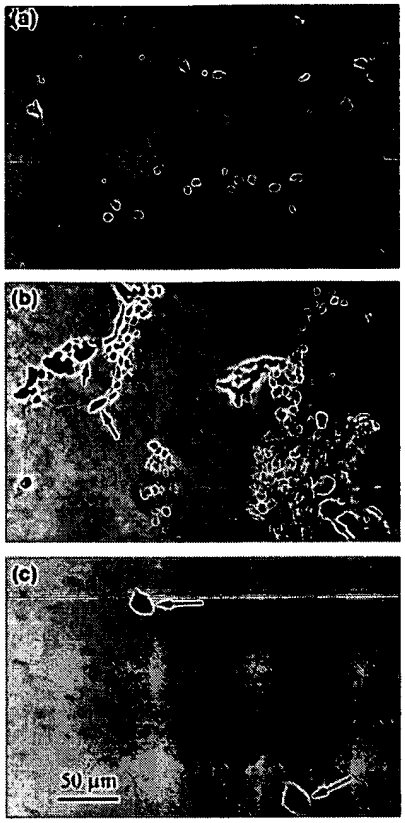

Fig. 2 Detection of proBDNF immunoreactivity in cell culture. (a) Cultured PC12 cells transfected with green fluorescence protein (GFP) plasmid. No significant immunoreactivity was detected. (b) Cultured PC12 cells transfected with proBDNF plasmid. A subpopulation of PC12 cells was intensely stained with the proBDNF antibodies. Arrows indicate positive cells. (c) Cultured dissociated adult dorsal root ganglia cells. Two cells were strongly labeled with proBDNF antibodies as indicated by arrows.

ProBDNF in peripheral tissues and endocrine organs

Table 1 summarizes the distribution of proBDNF in PNS and CNS examined by immunohistochemistry.

Skin

Strong immunoreactivity for proBDNF was localized in keratinocytes in epidermis (Fig. 4a,b). The intensity of the immunoreactivity was strongest in the basal layer and gradually reduced towards the skin surface (Fig. 4b). This localization pattern was similar to that of p75NTR and NGF after ventral root cut in our previous studies (Li *et al.* 2003). Immunoreactivity for proBDNF was also found in the subcutaneous layer mainly in nerve fibers running between sweat glandular cells (Fig. 4c, arrows).

Dorsal root ganglia

Immunoreactivity for proBDNF was present in a subpopulation of small to medium sized sensory neurons in DRG

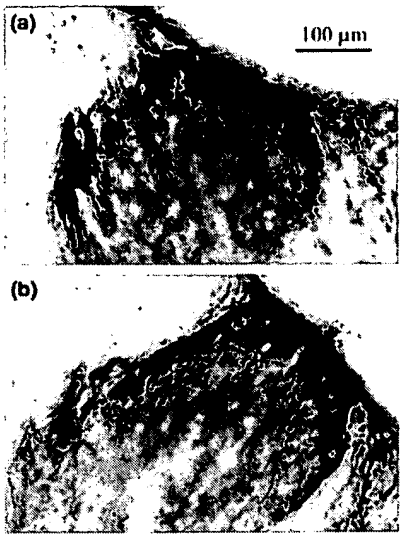

Fig. 3 Absorption test on immunostaining of the spinal cord for proBDNF. Spinal cord sections were incubated with proBDNF antibody in the absence (a) or presence (b) of 10 μg mature BDNF. There was no change in the specific staining in the dorsal horn when the antibody was absorbed by mature BDNF. Scale bar in (a) also applies to (b).

(Fig. 5a,b). Some axons and nerve fibers derived from immunoreactive cell soma were also immunoreactive (arrows in Fig. 5c). The immunoreactivity disappeared when DRG sections were incubated with preabsorbed antibodies.

Adrenal gland

Weak to moderate labeling was found in glomerular layer of cortex and medulla (data not shown).

Pituitary gland

Strong immunoreactivity for proBDNF was localized in a subpopulation of cells of the anterior lobe of the pituitary gland but not in the posterior lobe (Fig. 6a,b). The enlarged view showed that the immunoreactivity was present in many cells with short processes.

Olfactory epithelium

Moderate immunoreactivity was found in scattered cells in nasal olfactory mucosa (Fig. 6c,d).

Intestine

A few strongly immunoreactive cells for proBDNF were found in the small intestine. They were located in the myenteric plexus layer and in the mucosal and submucosal layers (data not shown).

ProBDNF in the spinal cord

Nerve terminals and fibers with strong immunoreactivity for proBDNF were found widely distributed in the spinal cord

Table 1 Localization of ProBDNF in adult rat

Location	Nerve terminals	Cell bodies
Skin	+	+ + +
Intestine	–	+
Adrenal gland	+	+ +
Pituitary gland	+	+ + +
Dorsal root ganglia	+	+ +
Superior cervical ganglia	+ +	+
Olfactory mucosa	–	+
Spinal cord dorsal horn	+ + +	+
Intermediolateral cell column	+ +	+ +
Central canal	+ +	–
Ventral horn	+	–
Medulla		
Nuclei tractus solitarius	+ + +	–
Hypoglossal nuclei	+	+
Spinal trigeminal nuclei	+ + +	+
Ventrolateral medulla	+ +	–
Inferior olive nuclei	+ +	+
Pons		
Cochlear nuclei	+	+
Vestibular nuclei	+	+
Raphe pontis nucleus	+ +	+
Spinal trigeminal nuclei	+ + +	+
Dorsal tegmental nuclei	+ +	+
Dorsal parabrachial nuclei	+ +	–
Midbrain		
Superior colliculus	+	+
Lateral genicular nuclei	+ +	+
Central gray	+ +	–
Interpeducular nuclei	+ + +	+
Substantia nigra	+ +	+
Zona incerta	+ + +	–
Cerebella		
Purkenjie layer	+	+
Granular layer	+ +	+
Thalamus	+	+
Hippocampus	+ +	+ +
Hypothalamus	+ + +	+
Amygdala	+ + +	+
Striatum	+	–
Septum	+ + +	+
Cortex	+ +	+ +
Basal forebrain	+ +	+
Olfactory bulb	+ +	+ +

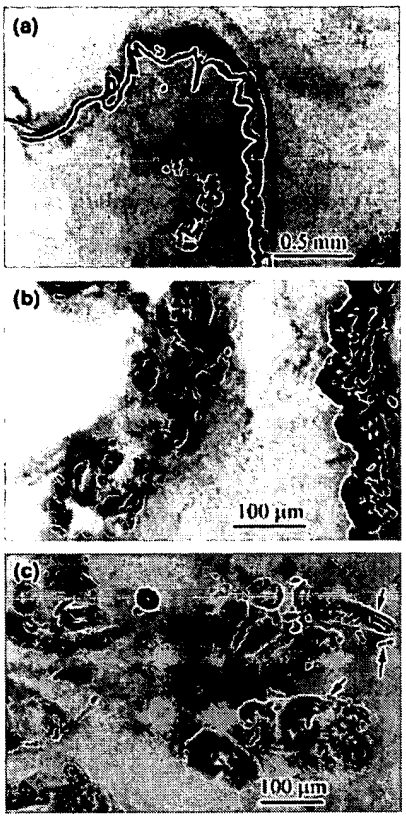

Fig. 4 Localization of proBDNF-like immunoreactivity in the footpad skin of the adult rat. (a) Strong immunoreactivity was detected in the basal layer of epidermis and moderate staining was found in sweat glands. (b) Enlarged view shows proBDNF was localized to keratinocytes with intensive staining at basal layer and reduced staining towards the skin surface. (c) Moderate staining was found in sweat gland. Arrows indicate nerve terminals immunoreactive for proBDNF.

(Fig. 7a). In particular, they are located in laminae I and II of the dorsal horn (Fig. 7c). Scattered positive fibers were also found in the deeper layers of the dorsal horn and in the ventral horn. Many immunopositive fibers were distributed around the central canal (Fig. 7d) and in the intermediolateral column (IML, Fig. 7b). Cell bodies in IML were also positive for ProBDNF. These may be preganglionic neurons supplying sympathetic neurons, as proBDNF nerve terminals were found in superior cervical ganglia around sympathetic neurons. Motor neurons and some neurons in gray matter were weakly labeled. All positive staining disappeared when the sections were incubated with the same antibodies preincubated with 10 μg/mL of immunizing peptide (Fig. 7f) and recombinant proteins (Fig. 7e). The staining was not affected if the sections were incubated in recombinant mature BDNF (Fig. 3b).

ProBDNF in the brain

Immunoreactivity for proBDNF was widely detected across all levels of the brain. Strong immunoreactivity was predominantly localized in nerve terminals and weak immunoreactivity was present in cell bodies (Table 1).

In the medulla (Fig. 8), intense proBDNF-immunoreactive terminals were present in spinal tract of trigeminal nerve (Fig. 8a,b) and central canal regions. The most intensely labeled structure for proBDNF was nuclei tractus solitarius (NTS)(Fig. 8a,c). Significant labeling of proBDNF in nerve

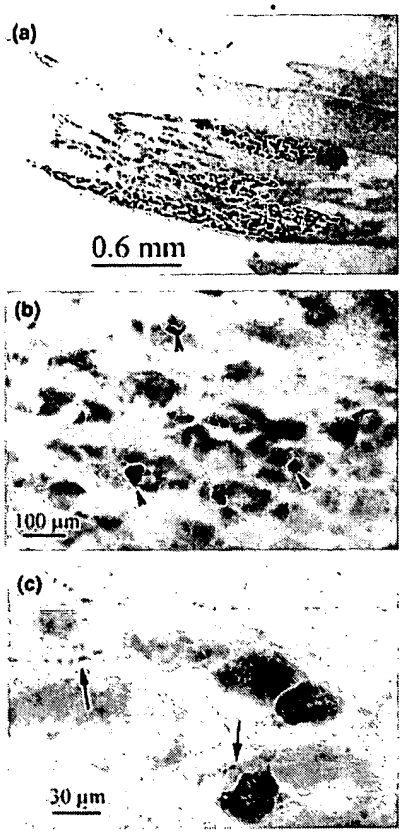

Fig. 5 ProBDNF-like immunoreactivity in adult rat DRG. (a) A lower magnification showed a subpopulation of DRG neurons immunoreactive for proBDNF; (b,c) higher magnifications show that some small neurons were immunoreactive for proBDNF. Arrowheads in (b) indicate positive neuron soma; arrows in (c) indicate positive axons.

terminals was also found in reticular nuclei (Fig. 8d). Immunoreactive nerve terminals were also found in the caudal and rostral ventrolateral medulla and inferior olivary nuclear complex. The staining in these regions can be abolished by preincubation of antibodies with peptide (Fig. 8f) and recombinant proBDNF (Fig. 8e).

In the pons, proBDNF immunoreactive nerve terminals were present in raphe pontine nucleus, dorsal tegmental nuclei, dorsal parabrachial nuclei, sensory trigeminal nuclei, facial nuclei and vestibular-cochlear nuclei (Table 1).

In the midbrain (Fig. 9), significant ProBDNF immunoreactivity was detected in nerve terminals of periaquaductal gray matter (Fig. 9d). Intense labeling of nerve terminals and weak labeling of cell bodies were present in the interpeduncular nucleus (Fig. 9b). Moderate immunoreactivity for proBDNF was detected in lateral geniculate nuclei (Fig. 9c). As shown in higher magnification (Fig. 9c), both nerve terminals and cellular components were positive for ProBDNF. Moderate immunostaining was present in superior gray layer of superior colliculus.

In the mesencephalon and thalamus, strong proBDNF immunoreactive nerve terminals were present in zona incerta, substantia nigra and central gray areas. Scattered proBDNF imunoreactive nerve terminals were found in most nuclei of the thalamus. Intense proBDNF labeling was found in all nuclei of hypothalamus (Fig. 10a), including paraventricular, dorsomedial hypothalamic nucleus (Fig. 10c), ventromedial hypothalamic nucleus (Fig. 10b), arcuate nuclei (Fig. 10a,b). The most intensely labeled structure was the medial eminence (Fig. 10d, ME). The immunoreactivity was mainly present in the nerve terminals and neuropil where staining was strong and diffuse.

Both nerve terminals and cell bodies in all regions of the hippocampus were stained by proBDNF antibodies (Fig. 11). The immunoreactivity was present in neurons in the pyramidal layer (Fig. 11b,d) and nerve terminals in the molecular layer (Fig. 11d). In the dentate gyrus, strong immunoreactive cell bodies and nerve terminals were present in granular cell layer (Fig. 11a,c).

One of the most strongly labeled structures for proBDNF was the amygdaloid complex (Fig. 12a). Both nerve terminals and cell bodies were labeled. Strongly positive cell bodies and nerve terminals were found in central nuclei of amygdala (Fig. 12a,b). Immunoreactive nerve terminals and neuropil were found in the medial nucleus (Fig. 12c).

In the septal area of forebrain (Fig. 13a), a number of septal nuclei were immunoreactive for proBDNF. The immunoreactivity was predominantly present in nerve terminals and neuropil but some cell bodies were also immunoreactive. As shown in Fig. 13(a), strongly labeled areas were the medial and lateral bed nuclei of stria terminalis (Fig. 13b,c), medial preoptic area (Fig. 13d), lateral intermediate (Fig. 13e,h) and ventral (Fig. 13f) septal nuclei and septofimbrial nuclei (Fig. 13g).

ProBDNF immunoreactive nerve terminals and cell bodies were also found in the cerebral cortex of all regions. Sparse proBDNF nerve terminals were found in the striatum. Both nerve terminals and cell bodies in the olfactory bulb and basal forebrain were moderately labeled for proBDNF (Table 1).

Discussion

There are two important outcomes from this study. First, we have produced a polyclonal antibody against the proregion of BDNF molecule and established, by a variety of tests, that the antibody is specific for proBDNF; it does not recognize mature BDNF. Second, we have used this antibody to describe, for the first time, the localization and distribution of proBDNF in both the CNS and PNS. This knowledge is an essential part of exploring the physiological functions of proBDNF.

Localization of neurotrophin proteins has always been challenging for several reasons. Firstly, neurotrophins are

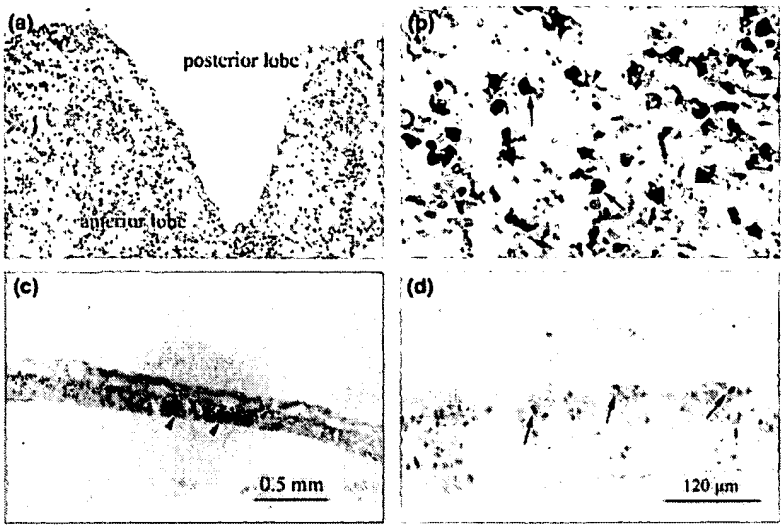

Fig. 6 Localization of proBDNF-like immunoreactivity in pituitary gland and in olfactory mucosa. (a,b) Lower and higher magnification microphotographs show proBDNF-like immunoreactivity in a subpopulation of cells of the anterior lobe of the pituitary gland. Arrows in (b) indicate positive cells. Arrowhead indicates cell with positive processes. (c,d) Lower and higher magnification microphotographs show proBDNF-like immunoreactivity in the olfactory mucosa. Arrowheads in (c) indicate epithelial neuronal cell layer. Arrows in (d) indicate positive cells in the neuronal cell layer.

synthesized in only limited quantities for their neurotrophic functions on target cells (Levi-Montalcini 1987). Second, they are diffusible factors which are transported away from where they are synthesized. Third, they may be complexed with their receptors or binding proteins which prevent recognition by antibodies (Zhou *et al.* 1994; Zettler *et al.* 1996). Fourth, their antigenicity is very sensitive to fixatives. Over-fixation can suppress their immunoreactivity in tissues (Finn *et al.* 1986). Finally, it is difficult to raise highly specific and strong affinity antibodies for immunohistochemical studies due to their highly conserved amino acid sequences among different species (Murphy *et al.* 1993). Despite these difficulties, a number of previous studies have successfully mapped the distribution and localization of

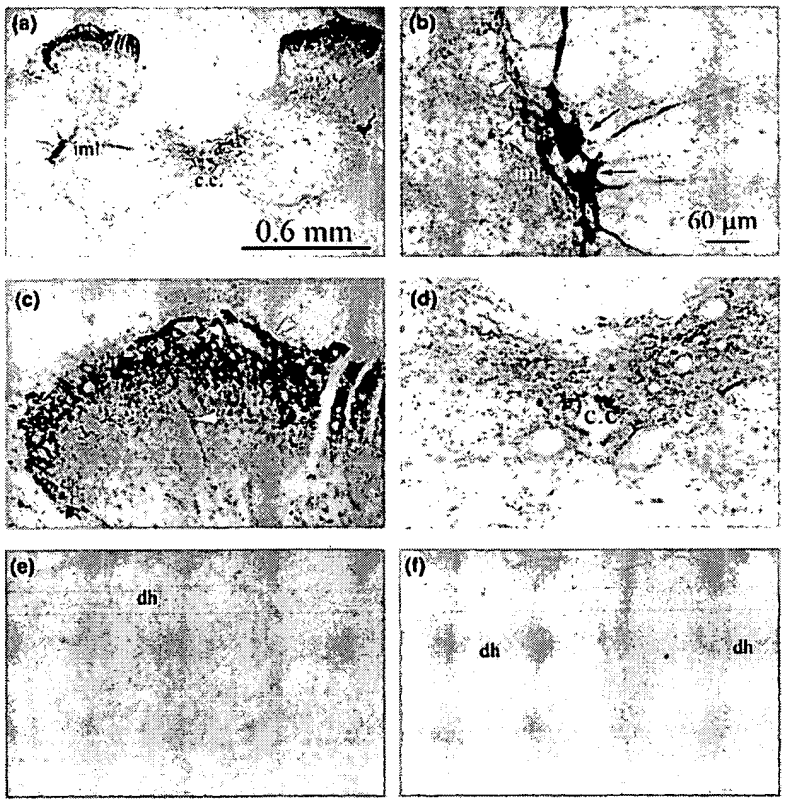

Fig. 7 Localization of proBDNF-like immunoreactivity in the spinal cord. (a) A lower magnification microphotograph shows the localization of proBDNF-like immunoreactivity in the spinal cord. (b–d) are higher magnification microphotographs showing proBDNF-like immunoreactivity in the regions of intermediolateral cell column (iml), dorsal horn (dh) and central canal (cc) of the spinal cord. (e) A microphotograph shows proBDNF-like immunoreactivity in the dorsal horn was completely absorbed by preincubation of the antibodies with recombinant proBDNF (10 μg/mL). (f) A microphotograph shows proBDNF-like immunoreactivity in the dorsal horn was completely absorbed by preincubation of the antibodies with immunizing peptide (10 μg/mL). Arrowheads indicate nerve terminals immunoreactive for proBDNF; dh: dorsal horn. Scale bar in (a) also applies to (f); scale bar in (b) also applies to (c,d,e).

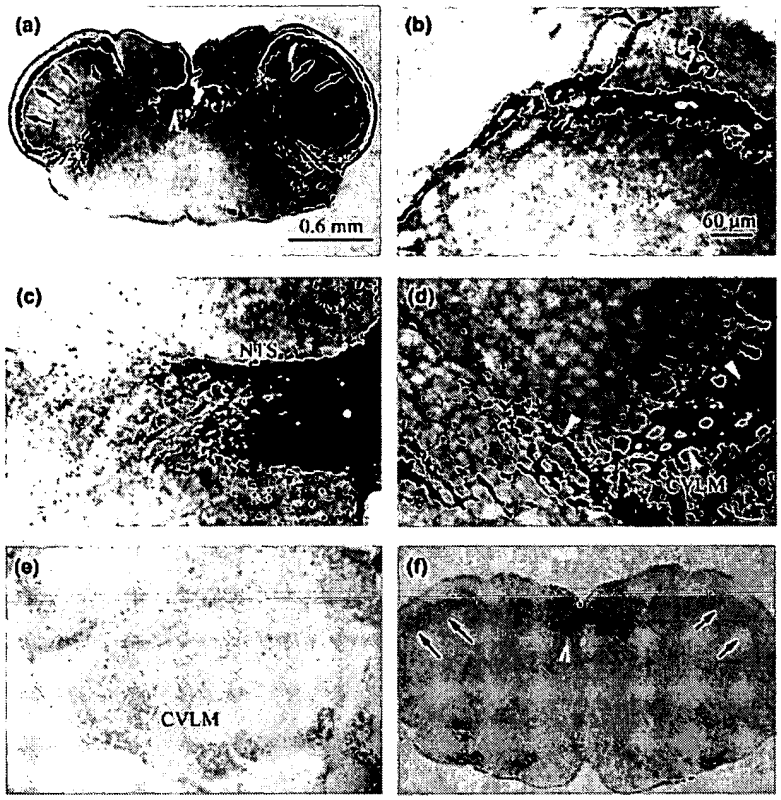

Fig. 8 Localization of proBDNF-like immunoreactivity in the medulla. (a) A low magnification micrograph of caudal medulla. Arrows indicate strong staining of spinal tract of trigeminal nerve (Spt5); arrowhead indicates nucleus tractus solitarius (NTS). (b–d) higher magnification microphotographs show proBDNF-like immunoreactivity in Spt5, NTS and caudal ventrolateral medulla (CVLM), respectively. Arrowheads in (d) indicate immunoreactive nerve terminals. (e) and (f) show that the immunoreactivity in two medulla sections was abolished by preabsorption with recombinant proBDNF (10 μg/mL) or immunizing peptide (10 μg/mL), respectively. Arrowhead and arrows in (f) indicate NTS and Spt5, respectively. Scale bar in (a) also applies to (f); scale bar in (b) also applies to (c) and (d).

mature BDNF (Wetmore *et al.* 1991; Conner *et al.* 1997; Yan *et al.* 1997).

Antibodies raised in different laboratories have different properties. The consensus indicates that BDNF is widely distributed in the brain and spinal cord, is anterogradely transported from cell bodies to nerve terminals (Zhou and Rush 1996a) and is stored in the releasing nerve terminals. These studies have provided anatomical evidence consistent with a role of BDNF as a neuromodulator on synaptic transmission and synaptic plasticity involving long-term potentiation (Kang and Schuman 1995), long-term depression (Ikegaya *et al.* 2002), neuropathic pain (Fukuoka *et al.* 2001), blood pressure control (Wang and Zhou 2002) and other physiological functions (Xu *et al.* 2003).

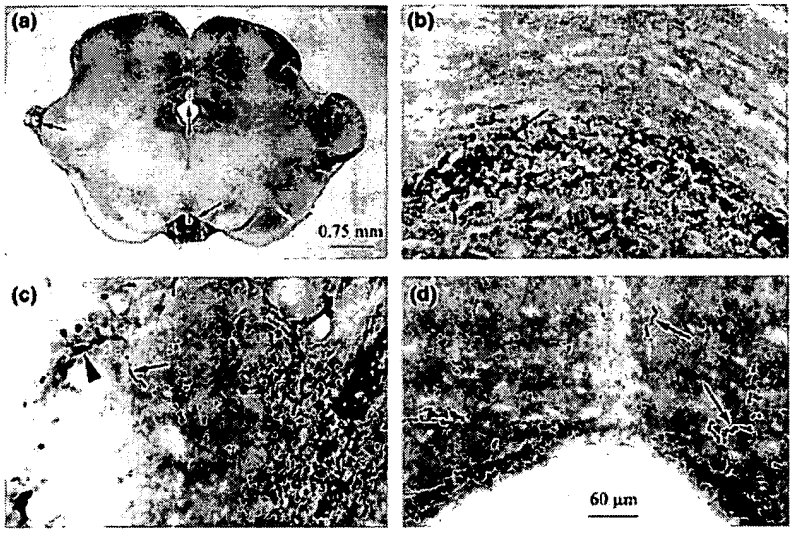

Fig. 9 Localization of proBDNF-like immunoreactivity in midbrain. (a) A lower magnification micrograph shows a section across the mid-brain. (b–d) show higher magnification microphotographs taken from the section shown in (a): (b) taken from b; (c) taken from c, (d) taken from d, as indicated by arrows also. Scale bar in (d) also applies to (b) and (c).

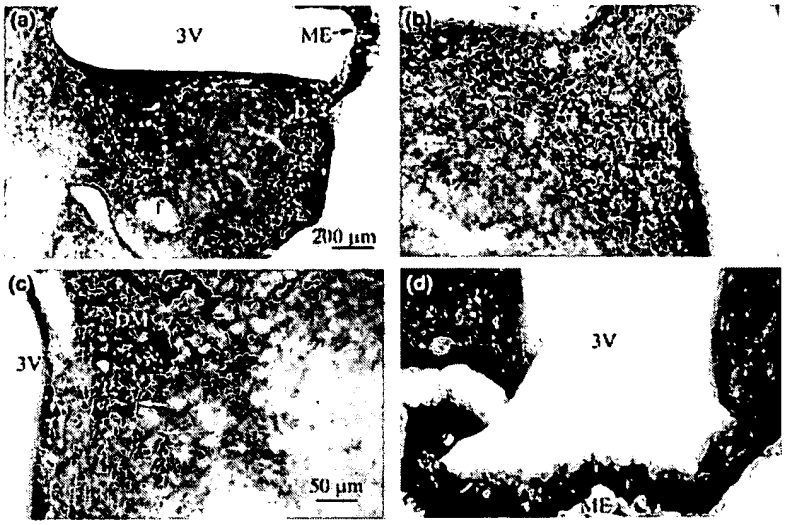

Fig. 10 Localization of proBDNF-like immunoreactivity in hypothalamus. (a) A low magnification micrograph shows strong staining in hypothalamic nuclei. 3V, third ventricle; f, fornix; ME, medial eminence. (b) A high magnification micrograph shows details of staining in the area marked by b in (a). Arc, arcuate hypothalamic nucleus; VMH, ventromedial hypothalamic nuclei. (c) A high magnification micrograph shows area of dorsal medial hypothalamic nucleus (DM). Arrows indicate positive nerve terminals. (d) A high magnification view shows strong staining in medial eminence (ME). Scale bar in (c) also applies to (b) and (d).

To examine the localization of proBDNF, we raised polyclonal antibodies in rabbits. To address the crucial issue of antibody specificity, we characterized the antibodies exhaustively. First, we chose the unique sequence in the proBDNF region which has no homology with known sequences of other proteins in mammals, to avoid potential cross-reaction of generated antibodies to other molecules. Second, we made recombinant proBDNF in *E. coli* and showed that the antibodies detected exogenous recombinant with a molecular weight corresponding to proBDNF. The detected heavy band was abolished by preincubation of the antibodies with the peptide. Third, we stained PC-12 cells transfected with proBDNF plasmid and DRG neurons cultured *in vitro*, which express a high level of BDNF. The result showed the antibodies intensely labeled transfected cells and a subpopulation of cultured DRG neurons. Fourth, the staining presented here was completely abolished by preincubation with either immunizing peptide or recombinant proBDNF but not by mature BDNF, suggesting the specificity for proBDNF. Finally, western blot showed one major band, corresponding to the predicted 35 kDa size of the molecule, in the spinal cord, pituitary gland, DRG and hypothalamus where strong proBDNF-like immunoreactivity was detected. Detection of two bands in pituitary gland is not surprising as previous studies, using a radio labeling pulse-chase method in BDNF-viral transfected cells, found that proBDNF displays 32 and 28 kDa bands, which were possibly due to differential glycosylation or sulfation (Mowla *et al.* 2001).

Having established that the antibody that we had produced was specific for proBDNF, we used it to examine the localization and distribution of proBDNF in the brain and

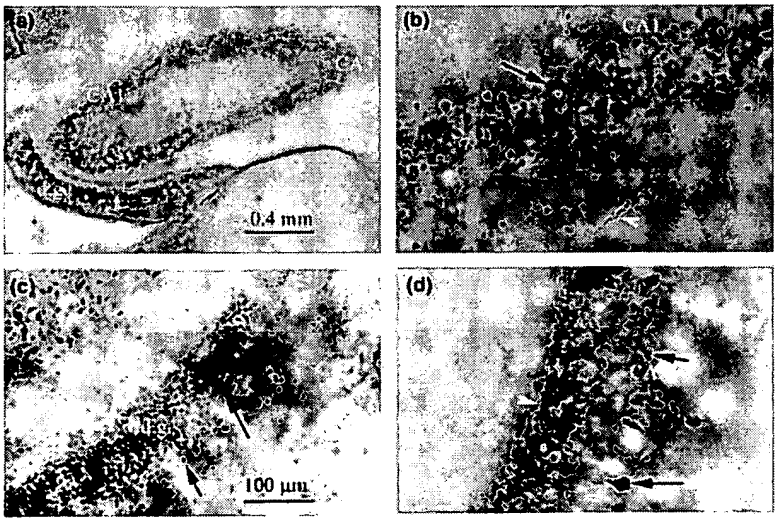

Fig. 11 Localization of proBDNF-like immunoreactivity in hippocampus. (a) A low magnification micrograph shows hippocampal region. d.g., dentate gyrus. (b–d) are high magnification micrographs taken from areas of CA1, dentate gyrus and CA3, respectively. Arrows indicate cell bodies immunoreactive for proBDNF; arrowheads indicate nerve terminals immunoreactive for proBDNF. Scale bar in (c) also applies to (c) and (d).

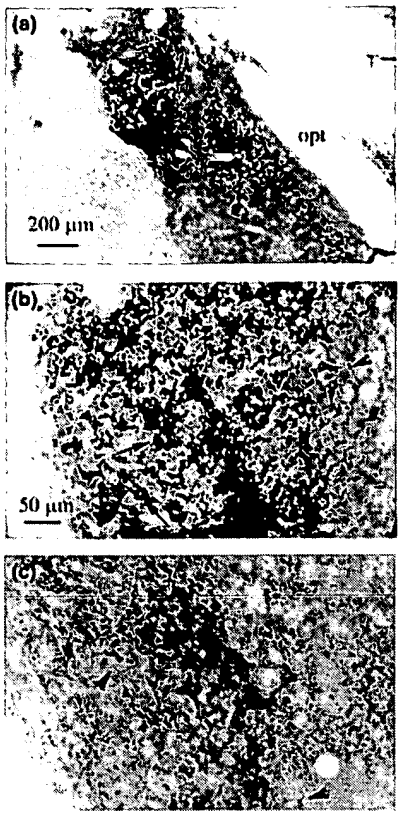

Fig. 12 Localization of proBDNF-like immunoreactivity in amygdaloid complex. (a) A low magnification micrograph shows amygdala nuclei. Ce, central nucleus; Me, medial nucleus; opt, optic nerve. (b) and (c) are high magnification micrographs showing central and medial nuclei of amygdala, respectively. Arrows indicate positive cell bodies; arrowheads indicate positive nerve terminals. Scale bar in (b) also applies to (c).

peripheral tissues. We found that proBDNF is present predominantly in nerve terminals in many brain regions, suggesting that, like mature BDNF, proBDNF may be also transported anterogradely. It is also possible that proBDNF in the nerve terminals is released from cell bodies and internalized by nerve terminals. However, this is unlikely as the immunohistochemical detection of internalized neurotrophins is restricted without acid treatment (Zhou *et al.* 1994). The distribution pattern of proBDNF is very similar to that of mature BDNF, with strongest labeling in superficial layers of the dorsal horn of the spinal cord, trigeminal nuclei, NTS, amygdaloid complex, hypothalamus and hippocampus (Wetmore *et al.* 1991; Conner *et al.* 1997; Yan *et al.* 1997). The distribution pattern suggests that previously mapped mature BDNF may also represent proBDNF, as antibodies raised against either peptide or recombinant molecule of BDNF will not only recognize mature BDNF but also the precursor. The antibody to proBDNF raised in the present study works well on tissues fixed in a higher concentration (4%) of paraformaldehyde. In addition, our preliminary data showed that this antibody recognizes proBDNF in tissues fixed with glutaraldehyde (data not shown). The characteristics of the antibody will allow us to localize proBDNF at the ultrastructural level.

There were, however, some distinct differences in the distribution of proBDNF compared with BDNF. ProBDNF was found in several tissues in which the presence of BDNF protein was not reported in earlier studies. For example, we found very strong proBDNF immunostaining in the basal layer of keratinocytes in the skin, strongly proBDNF-positive neurons scattered in the gut, in cells in the olfactory mucosa and in nerve terminals in the superior cervical ganglia.

The widespread distribution of proBDNF indicates that proBDNF may have important physiological functions in its own right in adult animals, in addition to the well-documented roles of mature BDNF. A previous study using a western blot technique with antibodies to mature BDNF also showed that in the human brain, proBDNF is the main form of BDNF gene product in all brain regions examined, including cortex, hippocampus, amygdala, nucleus basalis, cerebellum and thalamus (Michalski and Fahnestock 2003). As proBDNF is mainly localized in nerve terminals and in neuropil in rats as shown in the present study, proBDNF may be released alone or concomitantly with mature BDNF in an activity-dependent manner. The unprocessed proBDNF may participate in the regulation of synaptic plasticity and neurotransmission (Lu 2003b) and exert neurotrophic functions in the brain and spinal cord (Fahnestock *et al.* 2002). Recent studies showed that single amino acid mutation at codon 66 from valine to methionine in human causes impairment of short-term memory and changes in nerve activities in hippocampus and in the cortex as determined by brain imaging techniques (Egan *et al.* 2003; Hariri *et al.* 2003). Furthermore, the mutation results in dysfunction of the sorting of BDNF into releasing vesicles. The mutated molecule is mainly confined to cell bodies and is not transported into the nerve terminals. The activity-dependent release of mutated BDNF is also impaired (Egan *et al.* 2003). The distribution of proBDNF in nerve terminals and in endocrine glands suggests that proBDNF may be released to act as an endocrine, autocrine or paracrine factor.

In the present study, the western blot technique did not detect the enzymatically processed pro-region of BDNF. However, the lack of detection cannot exclude the presence of pro-region of BDNF in the nerve terminals. Theoretically, the antibodies used should recognize both full-length and pro-region of the molecule. Failure to show the band of the proregion fragment of the molecule suggests that the level of this molecule is very low or the sensitivity of our technique is below the detection limit.

Whether the full-length proBDNF or the fragment of the pro-region has any physiological functions is not known.

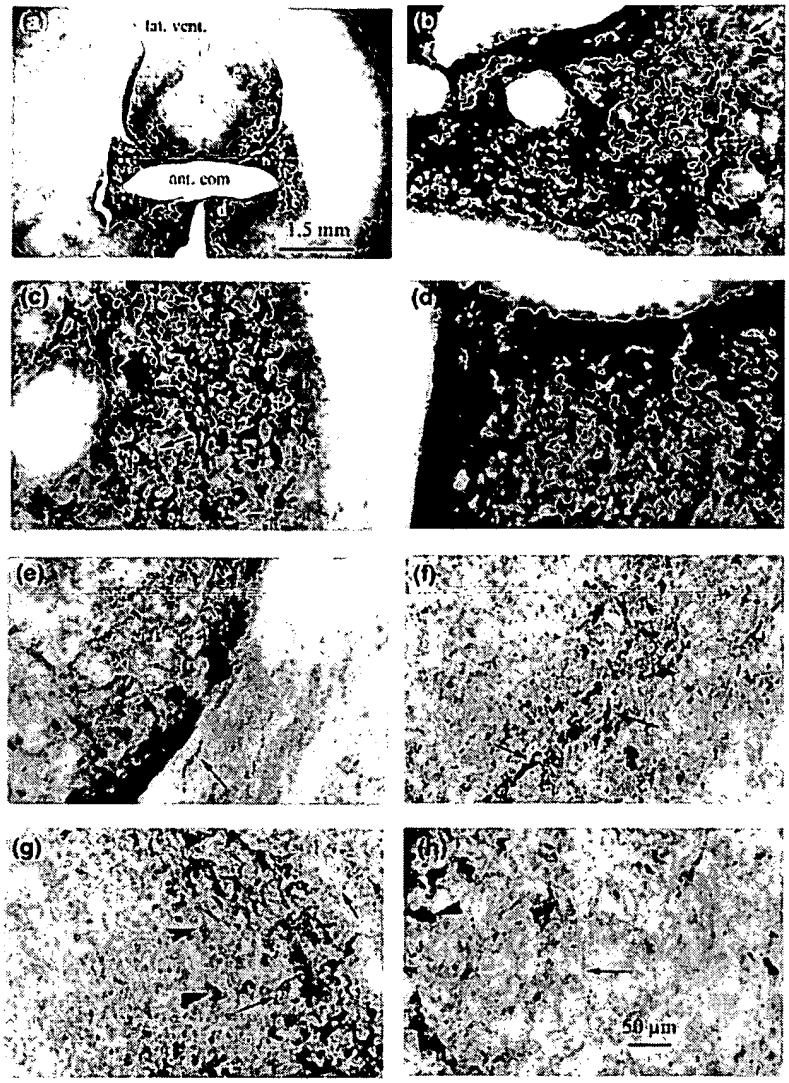

Fig. 13 Microphotographs show proBDNF-like immunoreactivity in coronal sections of septal region of the forebrain. (a) A lower magnification micrograph shows a section across the septum. Lat vent, lateral ventricle; ant. Com, anterior commissure. (b–h) are higher magnification microphotographs taken from areas marked by b, c, d, e, f, g and h, respectively, in (a). Scale bar in (h) also applies for panels (b–g). Arrows in (b,c,e–h) indicate positive nerve fibers. Arrowheads in (c), (g) and (h) indicate positive cell bodies.

ProNGF is released from transfected cells and binds to p75NTR and sortilin with very high affinity (higher than its affinity for binding to trkA) (Lee *et al.* 2001; Nykjaer *et al.* 2004). Furthermore, proNGF can trigger apoptosis of a variety of cells including PC12 cells and HEK293 cells *in vitro* and oligodendrocytes and corticospinal neurons *in vivo* after spinal cord injury and brain injury (Lee *et al.* 2001; Beattie *et al.* 2002; Harrington *et al.* 2004; Nykjaer *et al.* 2004). These studies suggest that the functions of neurotrophins are still far beyond our current understanding and that much further investigation on physiological and pathological functions of the neurotrophin precursors is needed.

Acknowledgements

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